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(54) Title: RANDOM AND TARGETED PROMOTER INTEGRATION FOR GENE ANALYSIS (57) Abstract A method of selectively altering the expression of a gene in a eukaryotic cell is disclosed. Expression of the gene can be manipulated at various points in development or in particular tissues. This method is particularly useful for observing the effect of altering the expression of an unknown gene or a gene whose function is unknown. The method can be used in eukaryotic cells <i>in vitro</i> or <i>in vivo</i> , for example by constructing transgenic eukaryotic organisms.		

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RANDOM AND TARGETED PROMOTER INTEGRATION FOR GENE ANALYSIS

This application claims the benefit of the following copending provisional applications: Serial No. 60/026,888, filed September 23, 1996, Serial No. 60/026,756, filed September 25, 1996, and Serial No. 60/033,793, filed December 30, 1996, each of which is incorporated by reference herein.

5

TECHNICAL AREA OF THE INVENTION

The invention relates to the area of eukaryotic gene function. More particularly, the invention relates to methods of analyzing and modulating the function of eukaryotic genes by genetic manipulation.

10

BACKGROUND OF THE INVENTION

Analysis of eukaryotic gene function is typically accomplished by examining the biological effects of defined mutations in known genes. Such mutations can be identified by phenotypic selection of mutant organisms or can be created by site-directed mutagenesis or homologous recombination targeting mutations to a particular gene of interest. For most mammalian genes, the rarity of targeted recombinants and phenotypically evident mutants limits the utility of these approaches. Thus, there is a need in the art for methods which can be used to observe eukaryotic, particularly mammalian, gene function.

15

SUMMARY OF THE INVENTION

It is an object of the invention to provide a eukaryotic cell for observing altered expression of a gene.

5 It is another object of the invention to provide a method of altering the expression of a gene in a eukaryotic cell.

It is yet another object of the invention to provide a transgenic eukaryotic organism.

It is an object of the invention to provide an insect cell for observing diminished expression of a target gene.

10 It is another object of the invention to provide a method of diminishing the expression of a target gene in an insect cell.

It is yet another object of the invention to provide a transgenic insect.

These and other objects of the invention are provided by one or more of the embodiments described below.

15 One embodiment of the invention provides a eukaryotic cell for observing altered expression of a gene. The cell comprises a first and a second DNA construct. The first DNA construct comprises an ectopic promoter. The ectopic promoter controls transcription of a gene in the eukaryotic cell. The second DNA construct comprises a regulatable promoter and a coding sequence for a foreign transcriptional transactivator. The regulatable
20 promoter controls the expression of the transcriptional transactivator. The transcriptional transactivator activates the ectopic promoter to alter expression of the gene.

Another embodiment of the invention provides a method of altering the expression of a gene in a eukaryotic cell. A first and a second DNA construct are introduced into the eukaryotic cell. The first DNA construct comprises an ectopic promoter. The ectopic
25 promoter controls transcription of a gene in the eukaryotic cell. The second DNA construct comprises a regulatable promoter and a coding sequence for a foreign transcriptional transactivator. The regulatable promoter controls the expression of the transcriptional transactivator. The transcriptional transactivator activates the ectopic promoter to alter expression of the gene.

Still another embodiment of the invention provides a transgenic eukaryotic organism. Cells of the transgenic eukaryotic organism comprise a first and a second DNA construct. The first DNA construct comprises an ectopic promoter. The ectopic promoter controls transcription of a gene in the cells of the organism. The second DNA construct comprises
5 a regulatable promoter and a coding sequence for a foreign transcriptional transactivator. The regulatable promoter controls the expression of the transcriptional transactivator. The transcriptional transactivator activates the ectopic promoter to alter expression of the gene.

One embodiment of the invention provides an insect cell for observing diminished expression of a target gene. The insect cell comprises a first and a second DNA construct.
10 The first DNA construct comprises a first promoter. The first promoter controls reverse orientation transcription of the target gene in the insect cell. The second expression construct comprises a second promoter and a coding sequence for a transcriptional transactivator. The second promoter controls the expression of the transcriptional transactivator. The transcriptional transactivator activates the first promoter whereby
15 expression of the target gene is diminished.

Another embodiment of the invention provides a method of diminishing the expression of a target gene in an insect cell. A first and a second DNA construct are introduced into the insect cell. The first DNA construct comprises a first promoter. The first promoter controls reverse orientation transcription of the target gene in the insect cell.
20 The second DNA construct comprises a second promoter and a coding sequence for a transcriptional transactivator. The second promoter controls the expression of the transcriptional transactivator. The transcriptional transactivator activates the first promoter, whereby expression of the target gene is diminished.

Still another embodiment of the invention provides a transgenic insect. Cells of the transgenic insect comprise a first and a second DNA construct. The first DNA construct
25 comprises a first promoter. The first promoter controls reverse orientation transcription of a target gene in the insect cell. The second DNA construct comprises a second promoter and a coding sequence for a transcriptional transactivator. The second promoter controls the expression of the transcriptional transactivator. The transcriptional transactivator

activates the first promoter, whereby expression of the target gene is diminished.

Another embodiment of the invention provides an insect cell for observing diminished expression of a target gene. The insect cell comprises a DNA construct comprising a promoter. The promoter controls reverse orientation transcription of the target gene in the insect cell.

Yet another embodiment of the invention provides a method of diminishing the expression of a target gene in an insect cell. A DNA construct comprising a promoter is introduced into the insect cell. The promoter controls reverse orientation transcription of the target gene in the insect cell, whereby expression of the target gene is diminished.

Even another embodiment of the invention provides a transgenic insect. Cells of the transgenic insect comprise a DNA construct. The DNA construct comprises a promoter. The promoter controls reverse orientation transcription of a target gene.

The present invention thus provides the art with a novel method of altering expression of a gene in a eukaryotic cell. The method can be used, *inter alia*, to observe the effects of altered and ectopic gene expression during development.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The inventors have discovered a method of selectively altering the expression of a gene in a eukaryotic cell. This method is particularly useful for observing the effect of altering the expression of an unknown gene or a gene whose function is unknown. The method can also be used for screening test compounds for the ability to alter gene expression in eukaryotes, for example, for agricultural purposes. The method can be used in eukaryotic cells *in vitro* or *in vivo*, for example by constructing transgenic eukaryotic organisms.

The method can be used to alter the expression of any gene in a eukaryotic cell, including the cells of protozoa, algae and other plants, fungi, sponges, coelenterates, worms, cephalopods, starfish, gastropods, arthropods, including insects, spiders, and crustaceans, and chordates, including vertebrates, especially amphibians, reptiles, birds, and mammals.

Genes involved in development, such as homeotic genes, are particularly suited for

use with the present method. Other eukaryotic genes, such as those which control apoptosis, signal transduction, enzymatic function, oncogenesis and metastasis, hormone function and regulation, motility, reproduction, and neuronal development and function, can also be observed effectively using the invention.

5 According to the method of the invention, expression of the gene can be manipulated at various points in development or in particular tissues. Alterations in gene expression induced by the method can be, for example, decreased or increased transcription, or temporal or spatial (tissue- or cell-specific) changes in transcription.

10 In order to alter expression of a gene, an ectopic promoter is integrated randomly into the genome of a eukaryotic cell. The ectopic promoter is a promoter which is adjacent to a gene which it does not normally regulate in the eukaryotic cell. The exogenous promoter is preferably a strong, regulatable promoter which can be controlled by a foreign trans-acting transcriptional transactivator, which is also introduced into the eukaryotic cell. The foreign transcriptional transactivator is one which is not normally present in the cell.
15 Transcription of the transcriptional transactivator is also preferably regulatable. Thus, when the transcriptional transactivator is not transcribed, the ectopic promoter is silent. In the presence of the transcriptional transactivator, however, the ectopic promoter is activated. Alternatively, the ectopic promoter is regulated by its tissue or developmental milieu. Thus, the promoter may be selectively activated or repressed in certain tissues or at certain stages
20 of development.

 If the ectopic promoter is integrated within the coding sequence of a gene, transcription of that gene can be disrupted and the phenotypic effects of that disruption observed. Alternatively, if the ectopic promoter is integrated near a gene (either 5' to an initiation codon or 3' to a termination codon) such that its presence does not interrupt the
25 coding sequence of the gene, expression of the gene can be altered by controlling the expression of the transcriptional transactivator which regulates the ectopic promoter. Similarly, if the ectopic promoter is tissue or developmentally regulated, it will be activated only under appropriate conditions in the appropriate tissue or developmental stage.

 According to one method, two DNA constructs are introduced into the eukaryotic

cell. The first DNA construct comprises an ectopic promoter. The ectopic promoter is a strong, regulatable promoter which can be controlled by a foreign trans-acting transcriptional activator. The ectopic promoter can integrate randomly into a coding sequence of a gene and interrupt its transcription, as discussed above. Alternatively, the
5 ectopic promoter is spatially or temporally regulated, as discussed above.

If integrated into the cell's DNA (within or 3' to the coding sequence) so that the orientation of the ectopic promoter is opposite to the orientation of the gene sequence and its native promoter, activation of the ectopic promoter can result in diminished expression of the gene. Diminished expression of the affected gene can be achieved by either or both
10 of two mechanisms. First, because transcription from the regulated promoter proceeds from the 3' end of the coding sequence, and transcription of the affected gene from its native promoter proceeds from the 5' end, concurrent transcription from the two promoters results in physical disruption of transcription (collision). While not wishing to be bound by any particular theory, we believe that both physical collision of the transcription machineries
15 and other mechanisms, such as the generation of positive superhelical tension by the RNA polymerase which initiates at the promoters, contribute to effect diminished expression of the affected gene.

Second, to the extent that reverse orientation transcription of the gene proceeds without disruption, an antisense transcript is produced. This antisense transcript can bind
20 to the coding sequence and prevent transcription of the gene. The antisense transcript can also bind to a pre-mRNA or mature mRNA transcript of the gene and prevent its translation.

The foreign transcriptional transactivator can be any protein which is not normally present in the eukaryotic cell and which is capable of binding the ectopic promoter and
25 activating transcription of a gene which has randomly come under the regulatory control of that promoter. A variety of transcriptional transactivators are known to those of skill in the art. Preferably, the transcriptional transactivator is LexA, Gal4, or a Tet-repressor-derived transcriptional activator such as the Tet-On™ "gene switch" (CLONTECH).

The second DNA construct comprises a regulatable promoter and a coding sequence

for the foreign transcriptional transactivator. The regulatable promoter can be a naturally occurring promoter or can be genetically engineered. The regulatable promoter can be a constitutively active promoter or can be a tissue-specific promoter, such as a promoter functional only in eye, muscle, heart, brain, lung, thymus, colon, stomach, liver, lymph node, blood, bladder, or ovary tissue. A cell type-specific promoter, for example, a promoter functional only in glia, neurons, T-cells, B-cells, or epithelial cells, can also be used to control the transcription of the transcriptional transactivator. Alternatively, the regulatable promoter can be a conditionally regulatable promoter, such as a temperature sensitive promoter or a promoter which is sensitive to a chemical inducer.

The DNA constructs can be fabricated as is known in the art, using standard recombinant DNA techniques. The DNA constructs can be flanked at each end by nucleotide sequences complementary to sequences in a cell's genomic DNA to facilitate integration of the expression constructs into the genome by homologous recombination. Preferably, at least the first DNA construct is integrated into the genome.

The DNA constructs can be introduced into a eukaryotic cell *in vitro* by any methods available in the art including, but not limited to, transferrin-polycation-mediated DNA transfer, transfection with naked or encapsulated nucleic acids, liposome-mediated cellular fusion, intracellular transportation of DNA-coated latex beads, protoplast fusion, viral infection, electroporation, and calcium phosphate-mediated transfection.

In one embodiment of the invention the DNA constructs are introduced into cells of a eukaryotic organism to create a transgenic organism. Techniques for constructing transgenic animals or plants are well known in the art.

In another embodiment of the invention, the method is used to inactivate or diminish the expression of a target gene in a cell, particularly an insect cell. The insect can be any member of the class *Insecta* in which further information regarding expression of a target gene is desired, including caterpillars, water nymphs and larvae, aphid-like insects, flealike insects, earwigs and silverfish, diving beetles and water bugs, hopperlike insects, plant bugs, toad bugs, and cockroaches, weevils, beetles, grasshoppers, crickets, and cicadas, mantids and walkingsticks, ants and termites, lacewings, dragonflies and damselflies, flies, bees,

wasps and hornets, moths, butterflies, ticks, mites, and scorpions. Preferably the insect is a fly, more preferably a *Drosophila melanogaster*.

5 The target gene can be any eukaryotic gene whose coding sequence is known and about whose expression more information is desired. Genes involved in insect development, such as homeotic genes, polycomb group genes, trithorax group genes, and genes involved in metamorphosis, are particularly suited for use with the present method. Other eukaryotic genes, such as those which control apoptosis, signal transduction, enzymatic function, oncogenesis and metastasis, hormone function and regulation, motility, reproduction, and neuronal development and function, can also be observed effectively using the invention.

10 The method is especially useful for observing the effects of altered expression of embryonic lethal genes in insects, such as *Ras*.

In order to control expression of the target gene, an endogenous or exogenous copy of the target gene coding sequence can be placed under the control of two promoters. One promoter is preferably the native promoter for the target gene and controls its transcription

15 in the normal 5' to 3' direction. The other promoter is a regulatable promoter which controls reverse orientation transcription of a gene (*i.e.*, transcription from the 3' end of the gene). The regulatable promoter is regulated by a transcriptional transactivator. Preferably the transcriptional transactivator is one which is not normally present in the cell. Transcription of the transcriptional transactivator is also preferably regulatable. Thus, when the

20 transcriptional transactivator is not transcribed, the regulatable promoter is silent and expression of the targeted gene proceeds normally. In the presence of the transcriptional transactivator, however, the regulated promoter is activated and, because of its reverse orientation with respect to the targeted gene, initiates transcription of the affected gene from the 3' end of the gene. The target gene with upstream and downstream promoters can be

25 introduced as a single cassette. Preferably one promoter is weaker and one is stronger. By regulating transcription from the stronger promoter, gene expression can be turned on or off, depending on whether the stronger promoter is 5' or 3'. Regulation of the promoter can be by either endogenous tissue-specific or stage-specific cues, or by introduced foreign transactivators.

According to one method, one or two DNA constructs can be introduced into the eukaryotic cell. The first DNA construct comprises a first promoter. The first promoter is a strong, regulatable promoter which can be controlled by a trans-acting transcriptional activator, or a tissue- or stage-specific cue. The DNA construct can be flanked at each end by nucleotide sequences complementary to sequences of the target gene or its flanking sequences, to integrate the first DNA construct at a desired site at, near, or in the target gene, as described below. If the orientation of the first promoter is opposite to the orientation of the target gene sequence and its normal orientation promoter, activation of the first promoter can result in diminished expression of the gene, as described above. The first DNA construct may also comprise the target gene coding sequence and a promoter 5' to it. The 5' promoter may be constitutive or regulatable.

The transcriptional transactivator can be any protein which is not normally present in the eukaryotic cell and which is capable of binding the first promoter and activating reverse orientation transcription of the target gene under the regulatory control of the first promoter. A variety of transcriptional transactivators are known to those of skill in the art. Preferably, the transcriptional transactivator is LexA, Gal4, or a Tet-repressor-derived transcriptional activator such as the Tet-On™ "gene switch" (CLONTECH).

The second DNA construct comprises a second promoter and a coding sequence for the transcriptional transactivator. Preferably, the second promoter is regulatable so that activation of the first promoter by the transcriptional transactivator can be controlled as desired. The second promoter can be a naturally occurring promoter or can be genetically engineered. The second promoter can be a constitutively active promoter or can be a tissue-specific promoter, such as a promoter functional only in eye, muscle, heart, brain, lung, thymus, colon, stomach, liver, lymph node, blood, bladder, or ovary tissue. A cell type-specific promoter, for example, a promoter functional only in glia, neurons, T-cells, B-cells, or epithelial cells, can also be used to control the transcription of the transcriptional transactivator. In one embodiment, the second promoter is an insect developmental stage-specific promoter, such as a nymph-, larval instar- or molt-specific promoter. Alternatively, the second promoter can be a conditionally regulatable promoter, such as a temperature

sensitive promoter or a promoter which is sensitive to a chemical inducer.

5 The first DNA construct can further comprise a coding sequence for the target gene and a third promoter. Preferably, the third promoter is the native promoter for the target gene *in situ*. The third promoter may also be regulatable, as discussed for the first promoter. The third promoter is located 5' to the coding sequence of the target gene and controls transcription of the target gene from 5' to 3'. The distance between the first and third promoters in the first DNA construct can be, for example, 10, 25, 40, 50, 75, 85, 100, 125, 150, 200, 250, 300, 500, 750, 1000, 1500, or 2000 nucleotides. By using combinations of stronger and weaker promoters as first and third promoters, the gene can be
10 selectively activated or repressed according to a desired cue.

According to another method, a DNA construct comprising a promoter is introduced into the cell such that the promoter integrates in or near a target gene in reverse orientation to the orientation of the target gene and its native promoter. The promoter can be a naturally occurring promoter or can be genetically engineered. It can be a constitutively
15 active promoter or can be regulatable, for example, a tissue-specific, cell type-specific promoter, or a developmental stage-specific promoter. A conditionally regulatable promoter, such as a temperature sensitive promoter or a promoter which is sensitive to a chemical inducer, can also be used to initiate reverse orientation transcription of the target gene.

20 The DNA constructs can be fabricated as is known in the art, using standard recombinant DNA techniques. The DNA constructs can be flanked at each end by nucleotide sequences complementary to sequences in a the target gene, to facilitate integration of the expression constructs into the target gene by homologous recombination. The length of the flanking sequences can vary, for example, from 6, 8, 10, 15, 25, or 50
25 nucleotides. Longer sequences may also be used. For integration into insect cell DNA, the DNA construct can be flanked by P elements, as is known in the art (see, for example, U.S. Patent 4,670,388, incorporated herein by reference). Preferably, at least the first DNA construct is integrated into the genome. Alternatively, the DNA constructs can remain episomal or on a minichromosome.

Transgenic insects can also be constructed according to the method of the invention. Preferably the transgenic insect is a *Drosophila melanogaster*. Techniques for constructing transgenic flies are also well known. For example, the first DNA construct comprising a promoter which will control reverse orientation transcription of a gene, can be injected into a *Drosophila melanogaster* embryo, resulting in a mosaic embryo which contains the first DNA construct in some of its cells. This method is described in U.S. Patent 4,670,388. Additionally after the embryo matures into a mosaic adult, it can be mated with a strain of *Drosophila melanogaster* which has been genetically engineered using standard techniques to express a transcriptional transactivator, for example, Gal4, LexA, or a Tet repressor-derived activator, under the control of a second, regulatable promoter. As disclosed above, activation of the second, regulatable promoter can result in the transcriptional transactivator's expression in either temporally or spatially restricted patterns. The offspring of the two strains of *Drosophila* will express the targeted gene in tissues or cells or at stages when the transcriptional transactivator is not expressed. In the presence of the transcriptional transactivator, the expression of the targeted gene in these transgenic *Drosophila* will be diminished, as described above.

The eukaryotic cell can have one or two wild-type endogenous copies of the target gene or can lack wild-type endogenous copies of the target gene. Those of skill in the art will recognize that endogenous copies of the target gene can be mutated or deleted using standard genetic manipulations, including targeted mutagenesis, successive matings of heterozygous strains, and other techniques known in the art. In such cells lacking any wild-type copies of the gene, only the collision mechanism is required to diminish expression of the target gene.

If the cell has an endogenous copy of the target gene, the first DNA construct can comprise an identifying marker for distinguishing expression of the target gene in the first DNA construct from expression of the endogenous copy of the target gene. For example, a sequence encoding an epitope which can be specifically bound by an antibody, such as a hemagglutinin tag, can be included in the first DNA construct so that expression of the target gene sequence in the construct can be detected immunocytochemically, as is known

in the art.

The following are provided for exemplification purposes only and are not intended to limit the scope of the invention which has been described in broad terms above.

5 **EXAMPLE 1**

This example illustrates the construction of *brahma* transgenes containing promoters which can be regulated by Gal4.

Promoters which can be regulated by Gal4 can be inserted into a P-element vector carrying the *white* gene and a coding sequence for the *brahma* gene. The *brahma* gene
10 sequence used for these constructs encodes a hemagglutinin (HA)-epitope tagged brahma protein under the control of the native *brahma* promoter. Three constructs can be made:
(1) a construct in which the regulated promoter is placed in opposing orientation 3' to the endogenous *brahma* promoter within an intron which is less than 1 kilobase from the endogenous *brahma* promoter, (2) a construct in which the regulated promoter is placed
15 in the same orientation as the endogenous *brahma* promoter and located 5' to the endogenous promoter, and (c) a construct in which the regulated promoter is placed in opposite orientation to the endogenous *brahma* promoter and 3' to the brahma polyadenylation site.

Brahma gene expression in insect cells can be detected using antibodies to brahma
20 protein. Expression of the *brahma* transgene can be detected using antibodies to HA. Depending on the specificity of the promoter which is used to regulate the expression of Gal4 in these cells, the effects of altered patterns of *brahma* gene expression in various cell types and at various times during the life of the fly can be studied.

25 **EXAMPLE 2**

This example demonstrates the generation of transgenic strains of *Drosophila melanogaster*.

A DNA construct, for example, one of the *brahma* constructs described in Example 1, above, can be injected into *Drosophila melanogaster* embryos, as is known in the art,

resulting in mosaic embryos. These mosaic embryos mature after two to three weeks into mosaic adults. Individual adults are then back crossed to a *white* strain of fly. The resulting heterozygous transformants can be mapped to the X, second, or third chromosomes within one to two generations.

5 Expression of the tagged *brahma* protein can be measured throughout the developing embryo. Transformants can be crossed to various Gal4 driver lines of flies, as are known in the art, such as lines which express Gal4 in stripes in the developing embryo, under regulatory control of the engrailed or even-skipped promoter. These flies express Gal4 along the anterior-posterior axis of the developing embryo in fourteen and seven stripes,
10 respectively.

 Expression of Gal4 can be monitored using antibodies against Gal4. The resulting effect of expression of the *brahma* transgene can be measured with a monoclonal antibody against the HA protein, as previously described. Endogenous *brahma* expression can be measured using antibodies which specifically bind to the *brahma* protein. Double-label
15 immunofluorescence can reveal if activation of Gal4 interferes with *brahma* expression. A convenient internal control for this experiment is that expression of *brahma* should not be altered in the regions between the stripes of Gal4-expressing cells.

20

SYNOPSIS OF THE INVENTION

1. A eukaryotic cell for observing altered expression of a gene, comprising:
 a first DNA construct comprising an ectopic promoter, wherein the
 ectopic promoter controls transcription of a gene in the eukaryotic cell; and
25 a second DNA construct comprising a regulatable promoter and a coding
 sequence for a foreign transcriptional transactivator, wherein the regulatable promoter
 controls the expression of the transcriptional transactivator and wherein the
 transcriptional transactivator activates the ectopic promoter, whereby expression of the
 gene is altered.

2. The eukaryotic cell of item 1 wherein the ectopic promoter is integrated 3' to a termination codon of a gene and wherein the ectopic promoter controls reverse orientation transcription of the gene.
3. The eukaryotic cell of item 1 wherein the ectopic promoter is integrated 5' to an initiation codon of a gene and wherein the ectopic promoter controls transcription of the gene.
4. The eukaryotic cell of item 1 wherein the foreign transcriptional transactivator is selected from the group consisting of Gal4, LexA, and a Tet-repressor-derived transactivator.
5. A method of altering the expression of a gene in an eukaryotic cell, comprising the steps of:
- introducing into the eukaryotic cell a DNA construct comprising an ectopic promoter, wherein the ectopic promoter controls transcription of a gene in the eukaryotic cell; and
- introducing into the eukaryotic cell a DNA construct comprising a regulatable promoter sequence and a coding sequence for a foreign transcriptional transactivator, wherein the regulatable promoter controls transcription of the transcriptional transactivator and the transcriptional transactivator activates the ectopic promoter, whereby expression of the gene is altered.
6. The method of item 5 wherein the ectopic promoter is integrated 3' to a termination codon of a gene and wherein the ectopic promoter controls reverse orientation transcription of the gene.
7. The method of item 5 wherein the ectopic promoter is integrated 5' to an initiation codon of a gene and wherein the ectopic promoter controls transcription of the gene.
8. The method of item 5 wherein the foreign transcriptional transactivator is selected from the group consisting of Gal4, LexA, and a Tet-repressor-derived transactivator.
9. A transgenic eukaryotic organism wherein cells of the transgenic

eukaryotic organism comprise:

a first DNA construct comprising an ectopic promoter, wherein the ectopic promoter controls transcription of a gene in the cells of the transgenic eukaryotic organism; and

5 a second DNA construct comprising a regulatable promoter and a coding sequence for a foreign transcriptional transactivator, wherein the regulatable promoter controls the expression of the transcriptional transactivator and wherein the transcriptional transactivator activates the ectopic promoter, whereby expression of the target gene is altered.

10 10. The transgenic eukaryotic organism of item 9 wherein the ectopic promoter is integrated 3' to a termination codon of a gene and wherein the ectopic promoter controls reverse orientation transcription of the gene.

11. The transgenic eukaryotic organism of item 9 wherein the ectopic promoter is integrated 5' to an initiation codon of a gene and wherein the ectopic promoter controls transcription of the gene.

15 12. The transgenic eukaryotic organism of item 9 wherein the foreign transcriptional transactivator is selected from the group consisting of Gal4, LexA, and a Tet-repressor-derived transactivator.

20 13. An insect cell for observing diminished expression of a target gene, comprising:

a first DNA construct comprising a first promoter, wherein the first promoter controls reverse orientation transcription of the target gene in the insect cell; and

25 a second DNA construct comprising a second promoter and a coding sequence for a transcriptional transactivator, wherein the second promoter controls the expression of the transcriptional transactivator and wherein the transcriptional transactivator activates the first promoter, whereby expression of the target gene is diminished.

14. The insect cell of item 13 wherein the first DNA construct further

comprises a coding sequence of the target gene and a third promoter, wherein the first promoter is located 3' to the coding sequence and wherein the third promoter is located 5' to the coding sequence, and wherein the orientation of the first promoter is opposite to the orientation of the third promoter and the coding sequence.

- 5 15. The insect cell of item 13 wherein the insect is a fly.
16. The insect cell of item 15 wherein the fly is a *Drosophila melanogaster*.
17. The insect cell of item 13 wherein the insect cell does not contain a native wild-type copy of the target gene.
18. The insect cell of item 13 wherein the first DNA construct is integrated
- 10 into the genome of the insect cell.
19. The insect cell of item 14 wherein the first DNA construct further comprises an identifying marker for distinguishing expression of the target gene in the first DNA construct from expression of an endogenous copy of the target gene.
20. The insect cell of item 13 wherein the transcriptional transactivator is
- 15 selected from the group consisting of Gal4, LexA, and a Tet-repressor-derived transactivator.
21. The insect cell of item 13 wherein the second promoter is a regulatable promoter.
22. A method of diminishing the expression of a target gene in an insect
- 20 cell, comprising the steps of:
- introducing into the insect cell a first DNA construct comprising a first promoter, wherein the first promoter controls reverse orientation transcription of the target gene in the insect cell; and
- introducing into the insect cell a second DNA construct comprising a
- 25 second promoter sequence and a coding sequence for a transcriptional transactivator, wherein the second promoter controls transcription of the transcriptional transactivator and the transcriptional transactivator activates the first promoter, whereby expression of the target gene is diminished.
23. The method of item 22 wherein the first DNA construct further comprises

a coding sequence of the target gene and a third promoter, wherein the first promoter is located 3' to the coding sequence and wherein the third promoter is located 5' to the coding sequence, and wherein the orientation of the first promoter is opposite to the orientation of the third promoter and the coding sequence.

- 5 24. The method of item 22 wherein the insect cell is a fly cell.
25. The method of item 24 wherein the fly cell is a *Drosophila melanogaster* cell.
26. The method of item 22 wherein the insect cell does not contain a native wild-type copy of the target gene.
- 10 27. The method of item 22 wherein the first DNA construct is integrated into the genome of the insect cell.
28. The method of item 23 wherein the first DNA construct further comprises an identifying marker for distinguishing expression of the target gene in the first DNA construct from expression of an endogenous copy of the target gene.
- 15 29. The method of item 22 wherein the transcriptional transactivator is selected from the group consisting of Gal4, LexA, and a Tet-repressor-derived transactivator.
30. The method of item 22 wherein the second promoter is a regulatable promoter.
- 20 31. A transgenic insect wherein cells of the transgenic insect comprise:
 a first DNA construct comprising a first promoter, wherein the first promoter controls reverse orientation transcription of a target gene in the insect cell;
 and
 a second DNA construct comprising a second promoter and a coding
25 sequence for a transcriptional transactivator, wherein the second promoter controls the expression of the transcriptional transactivator and wherein the transcriptional transactivator activates the first promoter, whereby expression of the target gene is diminished.
32. The transgenic insect of item 31, wherein the first DNA construct further

comprises a coding sequence for the target gene and a third promoter, wherein the first promoter is located 3' to the coding sequence and wherein the third promoter is located 5' to the coding sequence, and wherein the orientation of the first promoter is opposite to the orientation of the third promoter and the coding sequence.

- 5 33. The transgenic insect of item 31 wherein the transgenic insect is a fly.
34. The transgenic insect of item 33 wherein the fly is a *Drosophila melanogaster*.
35. The transgenic insect of item 31 wherein the insect cell does not contain a native wild-type copy of the target gene.
- 10 36. The transgenic insect of item 32 wherein the first DNA construct further comprises an identifying marker for distinguishing expression of the target gene in the first DNA construct from expression of an endogenous copy of the target gene.
37. The transgenic insect of item 31 wherein the transcriptional transactivator is selected from the group consisting of Gal4, LexA, and a Tet-repressor-derived
- 15 transactivator.
38. The transgenic insect of item 31 wherein the second promoter is a regulatable promoter.
39. An insect cell for observing diminished expression of a target gene, comprising a DNA construct comprising a promoter, wherein the promoter controls
- 20 reverse orientation transcription of the target gene in the insect cell.
40. The insect cell of item 39 wherein the insect is a fly.
41. The insect cell of item 40 wherein the fly is a *Drosophila melanogaster*.
42. The insect cell of item 39 wherein the DNA construct is integrated into the genome of the insect cell.
- 25 43. The insect cell of item 39 wherein the promoter is a regulatable promoter.
44. A method of diminishing the expression of a target gene in an insect cell, comprising the step of introducing into the insect cell a DNA construct comprising a promoter, wherein the promoter controls reverse orientation transcription of the target gene in the insect cell, whereby expression of the target

gene is diminished.

45. The method of item 44 wherein the insect is a fly.

46. The method of item 45 wherein the fly is a *Drosophila melanogaster*.

47. The method of item 44 wherein the DNA construct is integrated into the
5 genome of the insect cell.

48. The method of item 44 wherein the promoter is a regulatable promoter.

49. A transgenic insect wherein cells of the transgenic insect comprise a
DNA construct comprising a promoter, wherein the promoter controls reverse
orientation transcription of a target gene.

10 50. The transgenic insect of item 49 wherein the insect is a fly.

51. The transgenic insect of item 50 wherein the fly is a *Drosophila
melanogaster*.

52. The transgenic insect of item 49 wherein the DNA construct is
integrated into the genome of cells of the transgenic insect.

15 53. The transgenic insect of item 49 wherein the promoter is a regulatable
promoter.

54. A DNA construct for altering expression of a target gene in a eukaryotic
cell, comprising:

20 a first promoter, a coding sequence of the target gene, and a second
promoter, wherein the first promoter controls reverse orientation transcription of the
target gene in the cell, wherein the first promoter is located 3' to the coding sequence,
and wherein the second promoter is located 5' to the coding sequence, and wherein the
orientation of the first promoter is opposite to the orientation of the third promoter and
the coding sequence; wherein the first promoter is regulated.

25 55. The DNA construct of item 54 wherein the first promoter is regulated in
a tissue-specific manner.

56. The DNA construct of item 54 wherein the first promoter is regulated in a
developmental stage-specific manner.

57. The DNA construct of item 54 wherein the first promoter is regulated by a

transcriptional transactivator which is not endogenous to the eukaryotic cell.

58. A eukaryotic cell comprising the DNA construct of item 54.

59. The eukaryotic cell of item 58 further comprising a second DNA construct comprising a third promoter and a coding sequence for a transcriptional transactivator, wherein the third promoter controls the expression of the transcriptional transactivator and wherein the transcriptional transactivator activates the first promoter, whereby expression of the target gene is altered.

60. The eukaryotic cell of item 58 wherein the first promoter is a stronger promoter than the second promoter.

61. A DNA construct for altering expression of a target gene in a eukaryotic cell, comprising:

a first promoter, a coding sequence of the target gene, and a second promoter, wherein the first promoter controls reverse orientation transcription of the target gene in the cell, wherein the first promoter is located 3' to the coding sequence, and wherein the second promoter is located 5' to the coding sequence, and wherein the orientation of the first promoter is opposite to the orientation of the second promoter and the coding sequence; wherein the second promoter is regulated.

62. The DNA construct of item 61 wherein the first promoter is regulated in a tissue-specific manner.

63. The DNA construct of item 61 wherein the first promoter is regulated in a developmental stage-specific manner.

64. The DNA construct of item 61 wherein the first promoter is regulated by a transcriptional transactivator which is not endogenous to the eukaryotic cell.

65. A eukaryotic cell comprising the DNA construct of item 61.

66. The eukaryotic cell of item 65 further comprising a second DNA construct comprising a third promoter and a coding sequence for a transcriptional transactivator, wherein the third promoter controls the expression of the transcriptional transactivator and wherein the transcriptional transactivator activates the second promoter, whereby expression of the target gene is altered.

67. The eukaryotic cell of item 65 wherein the second promoter is a stronger promoter than the first promoter.

CLAIMS

1. An eukaryotic cell for observing altered expression of a gene,
comprising:
5 a first DNA construct comprising an ectopic promoter, wherein the
ectopic promoter controls transcription of a gene in the eukaryotic cell; and
a second DNA construct comprising a regulatable promoter and a coding
sequence for a foreign transcriptional transactivator, wherein the regulatable promoter
controls the expression of the transcriptional transactivator and wherein the
10 transcriptional transactivator activates the ectopic promoter, whereby expression of the
gene is altered.
2. A method of altering the expression of a gene in an eukaryotic cell,
comprising the steps of:
integrating into the DNA of the eukaryotic cell a first DNA construct
15 comprising an ectopic promoter, wherein the ectopic promoter controls transcription
of a gene in the eukaryotic cell; and
introducing into the eukaryotic cell a second DNA construct comprising
a regulatable promoter sequence and a coding sequence for a foreign transcriptional
transactivator, wherein the regulatable promoter controls transcription of the
20 transcriptional transactivator and the transcriptional transactivator activates the ectopic
promoter, whereby expression of the gene is altered.
3. A transgenic eukaryotic organism wherein cells of the transgenic
eukaryotic organism comprise:
a first DNA construct comprising an ectopic promoter, wherein the
25 ectopic promoter controls transcription of a gene in the cells of the transgenic
eukaryotic organism; and
a second DNA construct comprising a regulatable promoter and a coding
sequence for a foreign transcriptional transactivator, wherein the regulatable promoter
controls the expression of the transcriptional transactivator and wherein the

transcriptional transactivator activates the ectopic promoter, whereby expression of the gene is altered.

4. An insect cell for observing diminished expression of a target gene, comprising:

5 a first DNA construct comprising a first promoter, wherein the first promoter controls reverse orientation transcription of the target gene in the insect cell; and

10 a second DNA construct comprising a second promoter and a coding sequence for a transcriptional transactivator, wherein the second promoter controls the expression of the transcriptional transactivator and wherein the transcriptional transactivator activates the first promoter, whereby expression of the target gene is diminished.

5. A method of diminishing the expression of a target gene in an insect cell, comprising the steps of:

15 introducing into the insect cell a first DNA construct comprising a first promoter, wherein the first promoter controls reverse orientation transcription of the target gene in the insect cell; and

20 introducing into the insect cell a second DNA construct comprising a second promoter sequence and a coding sequence for a transcriptional transactivator, wherein the second promoter controls transcription of the transcriptional transactivator and the transcriptional transactivator activates the first promoter, whereby expression of the target gene is diminished.

6. A transgenic insect wherein cells of the transgenic insect comprise:

25 a first DNA construct comprising a first promoter, wherein the first promoter controls reverse orientation transcription of a target gene in the insect cell; and

a second DNA construct comprising a second promoter and a coding sequence for a transcriptional transactivator, wherein the second promoter controls the expression of the transcriptional transactivator and wherein the transcriptional

transactivator activates the first promoter, whereby expression of the target gene is diminished.

5 7. An insect cell for observing diminished expression of a target gene, comprising a DNA construct comprising a promoter, wherein the promoter controls reverse orientation transcription of the target gene in the insect cell.

8. A method of diminishing the expression of a target gene in an insect cell, comprising the step of introducing into the insect cell a DNA construct comprising a promoter, wherein the promoter controls reverse orientation transcription of the target gene in the insect cell, whereby expression of the target gene is diminished.

10 9. A transgenic insect wherein cells of the transgenic insect comprise a DNA construct comprising a promoter, wherein the promoter controls reverse orientation transcription of a target gene.

10. DNA construct for altering expression of a target gene in a eukaryotic cell, comprising:

15 a first promoter, a coding sequence of the target gene, and a second promoter, wherein the first promoter controls reverse orientation transcription of the target gene in the cell, wherein the first promoter is located 3' to the coding sequence, and wherein the second promoter is located 5' to the coding sequence, and wherein the orientation of the first promoter is opposite to the orientation of the third promoter and the coding sequence; wherein the first promoter is regulated.

20 11. A DNA construct for altering expression of a target gene in a eukaryotic cell, comprising:

25 a first promoter, a coding sequence of the target gene, and a second promoter, wherein the first promoter controls reverse orientation transcription of the target gene in the cell, wherein the first promoter is located 3' to the coding sequence, and wherein the second promoter is located 5' to the coding sequence, and wherein the orientation of the first promoter is opposite to the orientation of the third promoter and the coding sequence; wherein the second promoter is regulated.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 97/16893

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/85 C12N5/10 A01K67/033 C12N15/63 C12N15/67
C12N15/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A01K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BRAND A H ET AL: "TARGETED GENE EXPRESSION AS A MEANS OF ALTERING CELL FATES AND GENERATING DOMINANT PHENOTYPES" DEVELOPMENT, vol. 118, no. 2, 1993, pages 401-415, XP000674470	1-3
Y	see the whole document	4-11
X	VENTURA, A. M. ET AL.: "Silencing of human immunodeficiency virus long terminal repeat expression by an adenovirus Ela mutant" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA., vol. 87, February 1990, WASHINGTON US, pages 1310-1314, XP002055188 see the whole document	1

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *A* document member of the same patent family

Date of the actual completion of the international search

10 February 1998

Date of mailing of the international search report

05.03.98

Name and mailing address of the ISA

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 97/16893

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>YEH, E. ET AL.: "Green fluorescent protein as a vital marker and reporter of gene expression in Drosophila"</p> <p>PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA.,</p> <p>vol. 92, no. 15, July 1995, WASHINGTON US,</p> <p>pages 7036-7040, XP002055189</p> <p>see the whole document</p> <p>---</p>	1-3
X	<p>WO 93 23533 A (TSI CORP) 25 November 1993</p> <p>see page 13, line 6 - page 16, line 34</p> <p>see page 27, paragraph 3 - page 29, paragraph 3; claims</p> <p>---</p>	1-3
Y	<p>ELLEDGE, S. J. ET AL.: "Genetic selection for genes encoding sequence-specific DNA-binding proteins"</p> <p>PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA.,</p> <p>vol. 86, May 1989, WASHINGTON US,</p> <p>pages 3689-3693, XP002055190</p> <p>see the whole document</p> <p>-----</p>	4-11

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 97/16893

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
see FURTHER INFORMATION sheet PCT/ISA/210
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US 97/16893

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Remark : As far as claims 2, 5 and 8
are directed to a method of
treatment of the human/animal body , the search has been carried out and
based on the alleged effects of the compound/composition.

Information on patent family members

PCT/US 97/16893

Form PCT/ISA/210 (patent family annex) (July 1992)